Original Research Paper

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"Study of Gibberellic Acid Production by Submerged Fermentation Using Fusarium Moniliforme, Sheldon."

Microbiology

KEYWORDS : Fusarium moniliforme, Gibberellic acid, Submerged Fermentation, Wheat bran extract, Wheat flour, Maize flour

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ABSTRACT

Gibberellic acid production using Fusarium moniliforme, isolated from wilted sugarcane plant has been investigated by submerged fermentation (SMF). The gibberellic acid production of 195 mgm/ml was obtained on commercial wheat bran extract medium in 250 ml flasks after 168 hrs of incubation. The gibberellic acid production rate was about 0.6 to 0.9 mgm/ml/hr during 96 to 168 hrs. Different carbon sources namely sucrose, lactose, maltose, soluble starch, glycerol, wheat flour and maize flour were tested as an additional carbon source along with CWB extract at the concentration of 2.5% w/w or v/w base to observe its effects on gibberellic acid production. Soluble starch has been proved the best additional carbon source for gibberellic acid production, which yielded 1720 mgm/ml of gibberellic acid after 168 hrs of incubation. Similarly, various nitrogen sources namely NH4Cl, NH4NO3, (NH4)2SO4, (NH4) MoO4 and Urea were tested as an additional nitrogen source at the concentration of 7.0 mg % level in CWB extract. Urea was proved as the best nitrogen source which yielded 405 mgm/ml of gibberellic acid after 168 hrs of incubation. We have observed about 7.5-fold and 3.5-fold increase in gibberellic acid production upon addition of soluble starch and urea respectively, in CWB extract using Fusarium moniliforme.

INTRODUCTION:

Fusarium moniliforme is reported to produce Gibberellic acids (GAs) which are a group of diterpenoic acids that function as plant growth regulators influencing a range of developmental processes in higher plants including stem elongation, germination, dormancy, flowering, sex expression, enzyme induction and leaf and fruit senescence. The origin of research in to gibberellins can be traced to Japanese plant pathologists who were investigating the causes of the "bakanae" (foolish seedling) disease which seriously lowered the yield of rice crops in Japan, Taiwan and throughout the Asian continent (Kurosawa E. 1926).

The first paper on the cause of bakanae as published in 1898. By Shotaro Hori who demonstrated that the symptoms were induced by infection with a fungus belonging to the genes *Fusarium*, probably *Fusarium heterosporium* Nees. Subsequently, Elichi Kurosawa (1926) found that culture filtrates from dried rice seedlings caused marked elongation in rice and other sub-tropical grasses. He concluded that bakanae fungus secretes a chemical that stimulates shoot elongation, inhibits chlorophyll formation and suppresses root growth.

Teijiro Yabuta initiated work on the isolation of the active component using the fungal strains provided by Kurosawa. As a result, non-crystalline solid was obtained from the culture filtrate that stimulated the growth of rice seedlings. This compound was named gibberellin by Yabuta in 1935; the first use of the term "gibberellin" in the scientific literature.

In 1938, Yabuta and his associate Yusuke Sumiki finally succeeded in crystallizing a pale yellow solid to yield gibberellin A ($C_{22}H_{26}O_7$) and gibberellin B ($C_{19}H_{22}O_3$). The names were subsequently inter changed in 1941 and the original gibberellin A was found to be inactive.

In the United States, the first research on gibberellins began after the Second World War by a research unit at Camp Dietrick, Maryland. In 1950, John E. Mitchell reported optimal fermentation procedures for the fungus, as well as the effects of fungul extracts on the growth of bean (Viciafaba) seedlings (Mitchell & Angel 1951). At about the same time in the UK, a team of researchers at Akers Research Laboratories (ICI) isolated a new gibberellin which was given the name "Gibberellic acid". This compound had physical properties different from the Japanese gibberellin A (Curtis & Cross, 1954). Samples were exchanged between Stodola and Grove and "Gibberellic acid" and gibberellin–X were found to have identical chemical and physical properties and the name Gibberellic acid was accepted by both groups. A structure for Gibberellic acid was proposed in 1956 but later revised (Grove–1961).

Fermentation mechanism of GA production is very complex as it is a secondary metabolite.

Maddox and Richest (1977) studying production of gibberellic acid using a dairy waste as the basal medium in submerged fermentation concluded that filtered whey could be feasible basal medium for the production of gibberellic acid. A maximum yield of 750 mg/liter was obtained, and the only supplement required was Mg^{2+} ion at a final concentration of 10 mg/liter.

Kumar and Lonasane (1987) studied the gibberellic acid production by solid state fermentation (SSF) and submerged fermentation (SMF). The study indicated better productivity with the former technique.

Qian *et. al.* (1994) worked on the production of gibberellic acid (GA) by *F. moniliforme* in solid state culture in flask culture as wells as in horizontal rotary reactor. The highest production rate of Gibberellic acid was observed with 80% maize flour mixed with wheat bran.

Industrially GA is produced throughout the world by submerged fermentation (SmF) technique using the ascomycetous fungus *Gibberella fujikuroi* (recently named *Fusarium fujikuroi*) the perfect stage of *Fusarium moniliforme* (Bruckner and Blechschmidt,1991). The cost of GA has restricted its use to impede application for plant growth promotion, except for certain high value plants. Reduction in its production costs could lead to wider application fields (Shukla et al., 2005). The important factor in obtaining high yields of GA is the carbon source employed. Also, GA production, like that of several other secondary metabolites is initiated by the limitation (depletion) of nitrogen source from the medium (Shukla et al., 2003). Improvement of the microbial producer strain (through subjecting the genetic material to physical and chemical mutagenic agents) offers the greatest opportunity for cost reduction without significant capital outlay (Stanbury et al., 1995; Lotfy et al., 2007). This

is achieved when a selected strain can synthesize a higher proportion of the product using the same amount of raw materials.

MATERIALS AND METHODS :

1.1: PRODUCTION OF GA_3 BY SUBMERGED FERMENTATION:

The method described by Kumar and Lonsane (1987) was used for the study of production of GA_3 by submerged fermentation.

An extract of wheat bran medium used was prepared by adding 1.0 liter distilled water to WB medium containing 1.0 kg commercial wheat bran. The contents were agitated on shaker at 28 ± 1 °C for 2 hours and the extract was filtered through muslin cloth. Additional water was added to wheat bran held in muslin cloth until 1.0 liter extract was collected. Thus 1.0 gm commercial WB gave 1.0 ml extract. 50 ml extract was taken in 250 ml Erlenmayer flask and sterilized at 121 °C for 15 minutes and allowed to cool. The flasks were inoculated with 15 % inoculum, prepared by inoculating 10^6 spores / ml to Czapek Dox liquid medium (100 ml medium / 250 ml flasks) and grown on rotary shaker at 150 rpm speed at 30 °C for 24 hours. All flasks of WB extract medium inoculated with the culture were kept on shaker agitated at 150 rpm at 28 ± 1 °C. Every day one flask was used for the estimation of GA_3 concentration and cell dry weight measurement.

1.2: EXTRACTION OF GA_3 FROM FERMENTED BROTH:

The fermented broth was filtered through Whatman no – 1 filter paper and GA_3 from the filtrate was extracted at pH -2.5 using 2.0 N HCl by adding ethyl acetate in three stages to obtain 45 ml extract from 50 ml filtered broth. The extract was evaporated to about 5.0 ml .GA_3 was estimated by colorimetric technique and mycellial dry weight was determined.

1.3: ESTIMATION OF GA_3 BY SPECTROPHOTOMETRIC TECHNIQUE:

The concentration of GA_3 in ethyl extract was estimated using spectrophotometric method described by Berriso *et. al.* (2004) at 254 nm in a UV/Visible spectrophotometer (Elico make).

1.4: EFFECT OF DIFFERENT CARBON AND NITROGEN SOURCES ON THE PRODUCTION OF GA_3 BY SUBMERGED FERMENTATION:

The method described by Kumar and Lonsane (1987) and (1990) was used for the enrichment studies.

The effect of enrichment of commercial wheat bran with seven different carbon sources were studied at 25 % level (w/w or v/w) based on the weight of commercial wheat bran.

Water insoluble carbon sources viz. wheat flour and maize flour and water soluble carbon substrates such as soluble starch and glycerol were directly added in mineral salt solution before sterilization while heat sensitive carbon substrates such as sucrose, lactose and maltose were sterilized at 10 lb pressure for 15 minutes and were added to the sterilized wheat bran extract medium before inoculation.

For enrichment of nitrogen sources commercial wheat bran medium was enriched with 70 mg nitrogen from different sources per 100 gm commercial wheat bran. Desired weight of different nitrogen sources except urea were dissolved in mineral salt solution and then used for moistening wheat bran. The urea solution of desired concentration was sterilized by passing through a bacteriological filter and was added to the sterilized medium at the time of inoculation. GA_3 produced and mycellial dry weight were determined at every 24 hrs of incubation.

RESULTS AND DISCUSSIONS:

2.1 GA_3 PRODUCTION BY SMF USING WB-EXTRACT MEDIUM :

GA_3 production by submerged fermentation studied and the results obtained are shown in Table 1. The GA_3 production was observed from 96 hrs onwards. In the initial three days the production was negligible and not detectable. From 96 hrs onwards the production increases at a higher rate up to 168 hrs then the rate of production decreases and the production was almost same after 168 hrs and 192 hrs. These results are similar to those reported by Kumar and Lonsane (1988). In their studies using *G. fujikuroi*, they found the similar pattern of GA_3 production as that of *F. moniliforme* observed in our studies. Samir A. Meleigy & Mahmoud A. Khalaf in their study in 2009 obtained 410 mg/ml of GA_3 using milk permeate medium. Doaa A.E.Sleem (2013) obtained 0.270 g/l of GA3 by submerged fermentation.

Table : 1 GA_3 production by submerged fermentation using *F. moniliforme* Sheldon .

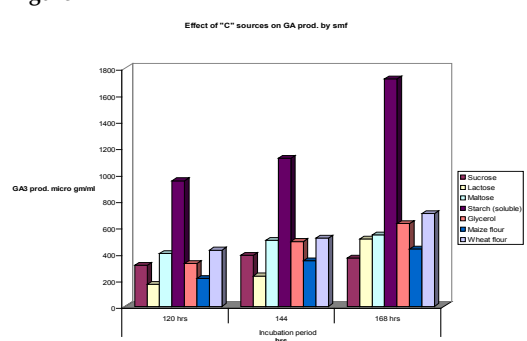
Incubation period in hrs.	Mycelial dry weight mg/ml	GA_3 production µg/ml of CWB extract
96	11.50	40
120	11.80	95
144	11.95	170
168	12.20	195
192	11.90	194

Note :(1) 5.0 ml ethyl extract was prepared from 50 ml WB extract medium (per flask) .

(2) 1.0 gm CWB gave 1.0 ml WB extract medium.

2.2 EFFECT OF DIFFERENT CARBON SOURCES ON GA_3 PRODUCTION BY SMF :

Figure - 1



The effect of seven different carbon sources on GA_3 production by smf was studied. The results obtained is shown in figure 1. In case of smf the soluble starch was found to be better source. It gave maximum production of GA3, 1720 µg / ml of CWB extract after 168 hrs. Also the rate of production of GA_3 was maximum between 144 hrs and 168 hrs. The second best source was wheat flour, but the amount of GA_3 produced with this source is nearly half of that obtained with the soluble starch. Among disaccharides Maltose gave good production of GA_3 followed by Sucrose and Lactose. Although Lactose was found to be poor source on the 120 hrs, gave good production on the 168 hrs. Kumar and Lonsane, 1989) reported that low glucose concen-

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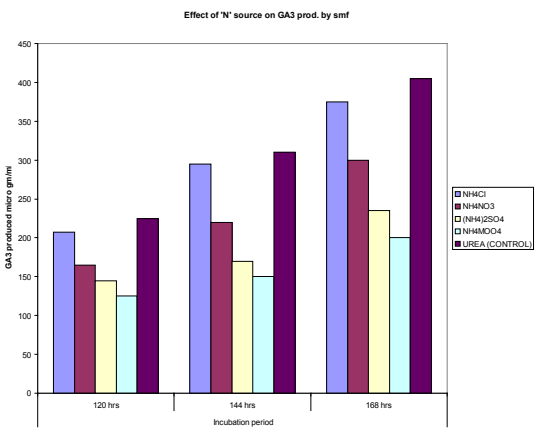
tration (< 4%) is necessary for the production of gibberellic acid and maintenance of biomass in the production phase. Rangaswamy, (2012) reported that of all the carbon sources, sucrose was the best carbon source under optimized conditions for gibberellic acid production. Gonzalez *et al*. (1994) found that the mixture of sucrose- starch was identified as the best carbon source for the GA3 production. Some oils such as sunflower oil and cooking oil have also been successfully used to enhance GA3 production (Gancheva *et al*., 1984 and Hommel *et al*., 1989). Doaa A.E.Sleem (2013) concluded fructose as the best carbon sourcefor GA3 production which yielded 0.609 g/l of GA3. Borrow *et al*. (1955) reported that, the yield of gibberellic acid was 40 mg/l when sucrose used as carbon source but it was lower when glucose used.

2.3 EFFECT OF DIFFERENT NITROGEN SOURCES ON GA3 PRODUCTION BY SMF :

The effect of different nitrogen sources on GA_3 production by smf were investigated and the results are shown in figure 2 .

Here urea proved to be better source of nitrogen compared to other ammonium compounds. The maximum yield of GA_3 obtained after incubation of 168 hrs was 405 µg / ml of CWB extract medium.

Figure 2



Amongst the ammonium salts studied NH_4Cl was found to be better source. It gave maximum GA_3 production, 375 µg / ml , after incubation of 168 hrs. The order of the effect of nitrogen sources on GA_3 productivity was NH_4Cl > NH_4NO_3 > $(NH_4)_2SO_4$ > $(NH_4)_2MOO_4$. Ammonium molibdate proved to be poor source. From the order of influence we may say that as the valency of ammonium ions increases the GA_3 productivity decreases. NH_4NO_3 gave less yield compared to NH_4Cl , this may be explained as , in case of former both NH_4^+ and NO_3^- may be competing with each other to be utilized as a nitrogen source. The use of nitrate ions may have an adverse effect on GA_3 production. Lale and Gadre, 2010 stated that the efficiency of gibberellic acid synthesis is obviously depends on the type of nitrogen source used and further they concluded that amongst all organic and inorganic nitrogen sources studied, the highest level of gibberellic acid was produced by *G. fujikuroi* mutant using wheat gluten as the nitrogen source. Doaa A.E.Sleem (2013) concluded yeast extract as the best nitrogen source for growth of *F. moniliforme* (8.62 g/l) but the maximum gibberellic acid production (0.797 g/l) was obtained when ammonium sulphate was used as sole nitrogen source. Kumar and Lonsane (1989) reported that production of 389 mg GA_3/ Kg dry matter-1 (mixed substrate containing coffee and cassava bagasse, 7:3 dry wt) was achieved with the saline solution, consisting of 10 mg of $(NH_4)SO_4$ 100 ml-1. Candau *et al.* (1992) showed that 1 g/l of NH_4NO_3 was the best concentration for gibberellic acid production.

SUMMARY:

Gibberellic acid production by submerged fermentation in commercial wheat bran extract medium was studied in in 250 ml flask on rotary shaker. The gibberellic acid production of 195 µgm/ml was obtained after 168 hrs of incubation. Soluble starch has been proved the best additional carbon source for gibberellic acid production, which yielded 1720 µgm/ml of gibberellic acid after 168 hrs of incubation. Similarly, urea was proved as the best nitrogen source which yielded 405 µgm/ml of gibberellic acid after 168 hrs of incubation. We have observed about 7.5-fold and 3.5-fold increase in gibberellic acid production upon addition of soluble starch and urea respectively, in CWB extract using *Fusarium moniliforme*.

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